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Establishment of a model experiment system to elucidate the mechanism by which
Zn-protoporphyrin is formed in nitrite-free dry-cured ham

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1 **Abstract**

2

3 The aim of this study was to establish a model experiment system to elucidate
4 the mechanism by which Zn-protoporphyrin (ZPP) is formed in Parma ham. The
5 established model consisted of myoglobin, meat and antibiotics, and incubation
6 under anaerobic conditions resulted in a greater yield of ZPP. Formation of ZPP
7 was observed even in the presence of various antiseptics. The amount of ZPP
8 formed increased as the period of incubation increased. ZPP formation was
9 inhibited by heating meat homogenate depending on the heating temperature.
10 Our results show that anaerobic conditions are suitable for the formation of ZPP
11 in meat products without nitrate or nitrite and that endogenous enzymes as well
12 as microorganisms may be involved in ZPP formation.

13

14 **Keywords:** Parma ham, Zn-protoporphyrin IX, heme, microorganism

15

1 1. Introduction

2

3 Salting have been used as a means of the meat preservation from ancient
4 times and various dry-cured meat products adapted to various climates have been
5 uniquely developed in various places over many years (Toldrá, 2002a; Flores,
6 1997). Nitrate and/or nitrite are now used in many meat products to improve
7 color. In Italian Parma ham, however, the use of nitrate or nitrite is banned.
8 Parma hams are manufactured by using only pork and salt (Toldrá, 2002b). The
9 inside color of the ham is bright red. The red pigment has not been identified
10 and, unlike known myoglobin derivatives, is stable to light (Morita, Niu, Sakata,
11 & Nagata, 1996). Parolari, Gabba and Saccani (2003) reported that dry curing of
12 meat without nitrate yields a lipophylic stable red pigment whose hydrophobicity
13 increases with ageing. We have recently identified the pigment extracted from
14 Parma ham as being Zn-protoporphyrin IX (ZPP), in which the iron in heme is
15 substituted by zinc (Wakamatsu, Nishimura, & Hattori, 2004).

16 Construction of a model experiment system would be useful for elucidation of
17 the mechanism by which ZPP is formed in Parma ham. Simple composition and
18 long incubation period would be important to model the manufacturing of Parma
19 ham. Composition of only meat and myoglobin is the simplest, but the effects of
20 microorganisms during a long incubation period can not be ignored. Morita, Niu,
21 Sakata and Nagata (1996) reported that the Parma ham pigment is formed by
22 staphylococci. Many studies have shown that microorganisms change the color
23 of meat and meat products (Kalchayanand, Ray, Field & Johnson, 1989;
24 Faustman Johnson, Cassens & Doyle, 1990; Arihara, Kushida, Kondo, Itoh,

1 Luchansky & Cassens, 1993). However, considering the low microbial counts
2 and the difficult conditions for microbial growth in the interior of dry-cured hams
3 (Toldrá & Etherington, 1988; Toldrá, 1998), it is unlikely that only
4 microorganisms contribute to the formation of ZPP in Parma ham. Additionally,
5 the oxygen pressure in the interior of meat products is the low oxygen pressure.
6 Effects of oxygen can not be ignored considering the high reactivity between heme
7 and oxygen. In this study, we developed a model experiment system to elucidate
8 the mechanism by which ZPP is formed during the process of traditional
9 dry-cured ham manufacturing, and we investigated the effect of oxygen on and
10 the involvement of microorganisms and endogenous enzymes in ZPP formation.

11

12

13 2. Materials and Methods

14

15 *2.1. Materials*

16

17 Pork loin samples were purchased from various retail markets. Myoglobin
18 (from horse skeletal muscle), penicillin G potassium, streptomycin sulfate,
19 gentamicin sulfate and sodium azide were purchased from Wako Pure Chemical
20 Industries, Ltd. (Osaka, Japan). Thimerosal was purchased from Nacalai Tesque,
21 Inc. (Kyoto, Japan). All other chemicals used in this study were of analytical
22 grade.

23 Penicillin and streptomycin were dissolved in sterilized distilled water at
24 10,000 units/ml and 10 mg/ml, respectively, and were kept frozen at -20 °C after

1 filtration sterilization until use. Gentamicin was dissolved in sterilized distilled
2 water at 10 mg/ml and was kept frozen at -20 °C after filtration sterilization until
3 use.

4

5 *2.2. Model experiment*

6

7 Model solutions consisted of myoglobin (final concentration of 0.1%) and pork
8 homogenates (final meat concentration of 20%) with or without preservatives.
9 About 20 grams of pork loin was homogenized with 2 volumes of distilled water
10 using a homogenizer (Cell Master CM-100, Az One Co., Tokyo, Japan) at 10,000
11 rpm for 1 min. Antibiotics were added to the model solutions to final
12 concentrations of 100 units/ml for penicillin G potassium, 0.1 mg/ml for
13 streptomycin sulfate and 0.05 mg/ml for gentamicin sulfate. Sodium azide and
14 thimerosal were added to the model solutions to final concentrations of 0.01%.
15 The solutions were put into gas-impermeable bags and incubated at 25 °C for 5
16 days. An anaerobic condition was obtained by using a commercial kit (Oxygen
17 Absorbing System; I.S.O. Inc., Yokohama, Japan) consisting of an oxygen absorber
18 (A-500HS, I.S.O. Inc.) and an oxygen indicator tablet. The color of the oxygen
19 indicator tablet changes from violet to pink when the oxygen concentration
20 becomes less than 0.1%.

21 For heat tests, pork homogenates and distilled water mixtures were heated in
22 a hot bath for 30 minutes after each setting temperature (40-80 °C) had been
23 reached. After cooling in water, myoglobin (final concentration of 0.1%) and
24 antibiotics (final concentrations of 100 units/ml for penicillin G potassium, 0.1

1 mg/ml for streptomycin sulfate and 0.05 mg/ml for gentamicin sulfate) were added
2 to the samples, and the samples were anaerobically incubated at 25 °C for 5 days.

3

4 *2.3. Fluorescent analysis*

5

6 One milliliter of incubated solution was mixed well with 3 ml of ice-cooled
7 acetone, and the mixture was held on ice for 30 minutes. After filtration through
8 filter paper (No.5C, Toyo Roshi Co., Ltd., Tokyo, Japan), the fluorescent spectra of
9 the extracts were measured from 500 to 700 nm at 420 nm for excitation using a
10 Model 650-60 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan)
11 (Wakamatsu, Nishimura, & Hattori, 2004). Fluorescence intensity at 590 nm for
12 excitation at 420 nm was regarded as the amount of ZPP formed. All operations
13 were carried out under shading as much as possible.

14

15 *2.4. Microbiological analysis*

16

17 One milliliter of model solution was mixed with 9 ml sterile
18 phosphate-buffered saline (PBS). Serial 10-fold dilutions were prepared by
19 diluting 1 ml in 9 ml sterile PBS. Two duplicate sheets were prepared from each
20 dilution by pouring 1 ml onto Sanita-kun Aerobic Count sheets (Chisso Corp.,
21 Tokyo, Japan) (Morita, Ushiyama, Aoyama, & Iwasaki, 2003). The sheets were
22 incubated at 37 °C for 48 h. Counts were determined from sheets bearing 30-300
23 colonies. Microbial counts are expressed in colony-forming units per milliliter
24 (CFU/ml).

1

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3 3. Results and discussion

4

5 Simple composition is desirable for the establishment of a model experiment
6 system to elucidate the mechanism of ZPP formation in Parma ham. The system
7 used in this study was composed of only 0.1% myoglobin and 20% pork. The
8 amount of added pork was determined from the ease of handling in preliminary
9 experiments. Since the oxygen partial pressure inside large-sized meat products
10 is remarkably low, we first investigated the influence of oxygen in our model
11 experiment system. Fluorescent spectra of 75% acetone extracts of model
12 solutions, consisting of only myoglobin and pork, after aerobic or anaerobic
13 incubation for 5 days at 25°C are shown in Fig. 1. When the model solution was
14 anaerobically incubated, strong and weak fluorescent peaks at about 590 and 640
15 nm were observed. This fluorescent spectrum is consistent with the ZPP
16 spectrum obtained in our previous study (Wakamatsu, Nishimura, & Hattori,
17 2004). On the other hand, strong fluorescence with a maximum peak at <500 nm
18 was emitted when the solution was aerobically incubated. A fluorescent peak
19 was observed at about 590 nm, but they were overlapped. Although the strong
20 fluorescence with a maximum peak at <500 nm is unidentifiable, since it was not
21 observed in Parma ham (Wakamatsu, Nishimura, & Hattori, 2004), it is thought
22 that an anaerobic condition is suitable for ZPP formation.

23 Next, we examined the effect of incubation period. Model solutions that each
24 consisted of 0.1% myoglobin and 20% pork were anaerobically incubated at 25 °C

1 for various periods, and then fluorescent intensities (Ex/Em 420/590 nm) of 75%
2 acetone extracts of model solutions were measured (Fig. 2). ZPP was not formed
3 after 1-day incubation, but the amount of ZPP dramatically increased thereafter.
4 The amount of ZPP increased only gradually after the seventh day of incubation.
5 We therefore decided that 5-7 days was the most suitable period for incubation in
6 our model experiment system.

7 Since there is a possibility of microbiological effects in our experiment system,
8 we investigated the effects of antiseptics. Model solutions consisting of 0.1%
9 myoglobin, 20% pork and various antiseptics were anaerobically incubated for 5
10 days at 25 °C. As shown in Fig. 3, the amount of ZPP formed in the presence of
11 thimerosal was the greatest and that in the presence of sodium azide was the
12 smallest. Formation of ZPP was observed in the presence of each of the
13 antiseptics used in this study. Many microorganisms survived after incubation
14 for five days without antiseptics ($>10^8$). However, in the presence of sodium
15 azide or antibiotics, the microorganisms did not survive after incubation. On the
16 other hand, the addition of thimerosal to the model solution did not result in
17 death of microorganisms after incubation (4.7×10^4). Sodium azide, which is one
18 of the respiratory and electron transport inhibitors, has been widely used as an
19 antiseptic in many biochemical experiments. However, since it bonds with heme
20 iron of many heme proteins, it may have some of influence on ZPP formation, i.e.
21 Zn-Fe substitution in heme. Thimerosal has also been widely used as an
22 antiseptic in many biochemical experiments, and it inactivates many enzymes by
23 bonding with thiol-groups. Although both are effective as antiseptics, their
24 antibacterial mechanism may have an effect on Zn-Fe substitution. Penicillin

1 blocks the formation of bacterial cell walls and has an antibacterial effect on
2 Gram-positive bacteria. Streptomycin and gentamycin inhibit initiation,
3 elongation and termination of protein synthesis in prokaryotes and induce
4 misreading. They have antibacterial effect on both Gram-negative and
5 Gram-positive bacteria. There is no cell wall in animal cells, and proteins are
6 not synthesized because meat is skeletal muscle after death. It is therefore
7 probable that these antiseptics have no effect on Zn-Fe substitution in our model.
8 Since these antibiotics act not bacteriostatically but bactericidally and since
9 microorganisms were not detected in model solutions after incubation for 5 days,
10 it appears that surviving microorganisms have little effect on Fe-Zn substitution.
11 We established a model experiment system that consists of meat, Mb and
12 antibiotics were anaerobically incubated.

13 Next, we examined the effects of oxygen on the formation of ZPP in the
14 presence of antibiotics. Fig. 4 shows fluorescent spectra of 75% acetone extracts
15 of model solution after anaerobic or aerobic incubation for 5 days at 25 °C. The
16 characteristic fluorescence spectrum pattern of ZPP was observed after anaerobic
17 incubation in the presence of antibiotics. On the other hand, little ZPP was
18 formed after aerobic incubation. The fluorescent intensities were remarkably
19 increased by removal of oxygen. These results suggest that oxygen inhibits the
20 formation of ZPP. Therefore, something other than microorganisms that induces
21 the formation of ZPP might exist in meat.

22 Since ZPP was formed in the presence of any of the antiseptics used in this
23 study, it is necessary to consider the effects of factors other than the action of
24 microorganisms. Therefore, we investigated the effects of pre-heating of meat on

1 ZPP formation. The amounts of ZPP formed at different heating temperatures
2 are shown in Fig. 5. In the control experiment, a considerable amount of ZPP
3 was formed after incubation for five days. On the other hand, the amount of ZPP
4 was reduced by heating at 40 °C for 30 minutes, and the amount of ZPP decreased
5 with rise in heating temperature. Heating at 60 °C or higher resulted in
6 equivalent degrees of fluorescence intensity before and after incubation and
7 almost no formation of ZPP. It is not clear whether fluorescent intensity is
8 reduced by heating at 40 °C, a temperature equivalent to body temperature.
9 Since formation of ZPP in Parma ham differs from the reaction in a living body, it
10 may be caused by unknown mechanism.

11 Zn is nonenzymatically inserted into porphyrins by reaction of a salt in an
12 acidic or basic medium (Sanders et al., 2000). However, the reduction in the
13 amount of ZPP formed by heating the meat homogenate is thought to be due to a
14 substitution reaction by protein, not metal ions. Microbial counts in the interior
15 of dry-cured hams are low, and conditions are not favorable for microbial growth
16 (Toldrá, 1998; Toldrá & Etherington, 1988). It is conceivable that the ZPP
17 formation from heme is caused by the action of meat endogenous enzymes.
18 Actually, it has been reported that enzymes catalyzing the formation of ZPP exist
19 in most organisms (Labbe, Vreman & Stevenson, 1999; Dailey & Dailey, 2003).
20 However, it is not clear whether the activities of those enzymes are maintained
21 during the long manufacturing process of Parma ham.

22 We established a model experiment system in which ZPP was formed by
23 incubating myoglobin, meat and antibiotics in the absence of oxygen. Since,
24 however, the antibiotics used have no effect on fungi and yeast, it is unlikely that

1 all microorganisms were controlled in this study. Further studies are needed to
2 elucidate the mechanism of Fe-Zn substitution during the manufacturing process
3 of Parma ham.

4

5

6 4. Conclusions

7

8 ZPP, a nitrate and/or nitrite-free dry-cured ham pigment, could be formed in a
9 simple model. Oxygen inhibited the formation of ZPP. The finding that ZPP
10 was formed even in the presence of antiseptics, the fact that microbial counts
11 inside hams are low and that the conditions are not favorable for microbial growth,
12 and the finding of reduction in the amount of ZPP formed by heating meat
13 indicate that not only microorganisms contribute to the formation of ZPP in
14 nitrate and/or nitrite-free dry-cured meat products.

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17 Reference

18 Arihara, K., Kushida, H., Kondo, Y., Itoh, M., Luchansky, J. B., & Cassens, R. G.

19 (1993). Conversion of metmyoglobin to bright red myoglobin derivatives by

20 *Chromobacterium violaceum*, *Kurthia* sp., and *Lactobacillus fermentum*

21 JCM1173. *Journal of Food Science*, 58, 38-42.

22 Dailey, H. A., & Dailey, T. A. (2003). Ferrochelatase In K. M. Kadish, K. M. Smith,

23 & R. Guilard, *The Porphyrin Handbook* (Vol. 12, pp. 93-121). San Diego:

24 Academic Press.

- 1 Faustman, C., Johnson, J. L., Cassens, R. G., & Doyle, M. P. (1990). Color
2 reversion in beef. Influence of psychrotrophic bacteria. *Fleischwirtschaft*, *70*,
3 676-679.
- 4 Flores, J. (1997). Mediterranean vs northern European meat products. Processing
5 technologies and main differences, *Food Chemistry*, *59*, 505-510.
- 6 Kalchayanand, M., Ray, B., Field, R. A., & Johnson, M. C. (1989). Spoilage of
7 vacuum-packaged refrigerated beef by *Clostridium*. *Journal of Food*
8 *Protection*, *52*, 424-426.
- 9 Labbe, R. F., Vreman, H. J., & Stevenson, D. K. (1999). Zinc protoporphyrin: A
10 metabolite with a mission, *Clinical Chemistry*, *45*, 2060-2072.
- 11 Morita, H., Niu, J., Sakata, R., & Nagata, Y. (1996). Red pigment of Parma ham
12 and bacterial influence on its formation. *Journal of Food Science*, *61*,
13 1021-1023.
- 14 Morita H., Ushiyama M., Aoyama S. & Iwasaki M. (2003). Sensitivity and
15 specificity of the Sanita-kun Aerobic Count: Internal validation and
16 independent laboratory study, *Journal of AOAC International*, *86*, 355-366.
- 17 Parolari, G., Gabba L., & Saccani G. (2003). Extraction properties and absorption
18 spectra of dry cured hams made with and without nitrate, *Meat Science*, *64*,
19 483-490.
- 20 Sanders, J. K. M., Bampos, N., Clyde-Watson, Z., Darling, S. L., Hawley, J. C.,
21 Kim, H.- J., Mak, C. C., & Webb, S. J. (2000). Axial Coordination Chemistry of
22 Metalloporphyrins. In K. M. Kadish, K. M. Smith, & R. Guillard, *The*
23 *Porphyrin Handbook* (Vol. 3, pp. 1-48). San Diego: Academic Press.
- 24 Toldrá, F. (2002a). A historical perspective. *Dry-cured meat products* (pp. 1-5).

- 1 Connecticut: Food & Nutrition Press.
- 2 Toldrá, F. (2002b). Manufacturing of dry-cured ham. *Dry-cured meat products* (pp.
3 27-62). Connecticut: Food & Nutrition Press.
- 4 Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured
5 meat products, *Meat Science*, *49*, S101-S110.
- 6 Toldrá, F. & Etherington, D. J. (1988). Examination of cathepsins B, D, H and L
7 activities in dry-cured hams, *Meat Science*, *23*, 1-7.
- 8 Wakamatsu, J., Nishimura, T., & Hattori, A. (2004). A Zn-porphyrin complex
9 contributes to bright red color in Parma ham. *Meat Science*, *67*, 95-100.

1 Figure & Table Legends

2

3 Fig. 1. Fluorescence pattern of acetone extract of model solution after
4 anaerobic (—) and aerobic (----) incubation for 5 days at 25 °C.

5

6 Fig. 2. Effects of anaerobic incubation time on the formation of ZPP.

7

8 Fig. 3. Effects of various antiseptics (sodium azide, 0.01%; antibiotics [100
9 units/ml penicillin G potassium, 0.1 mg/ml streptomycin sulfate and 0.05
10 mg/ml gentamicin sulfate]; thimerosal, 0.01%) on the formation of ZPP
11 after anaerobic incubation for 5 days at 25 °C.

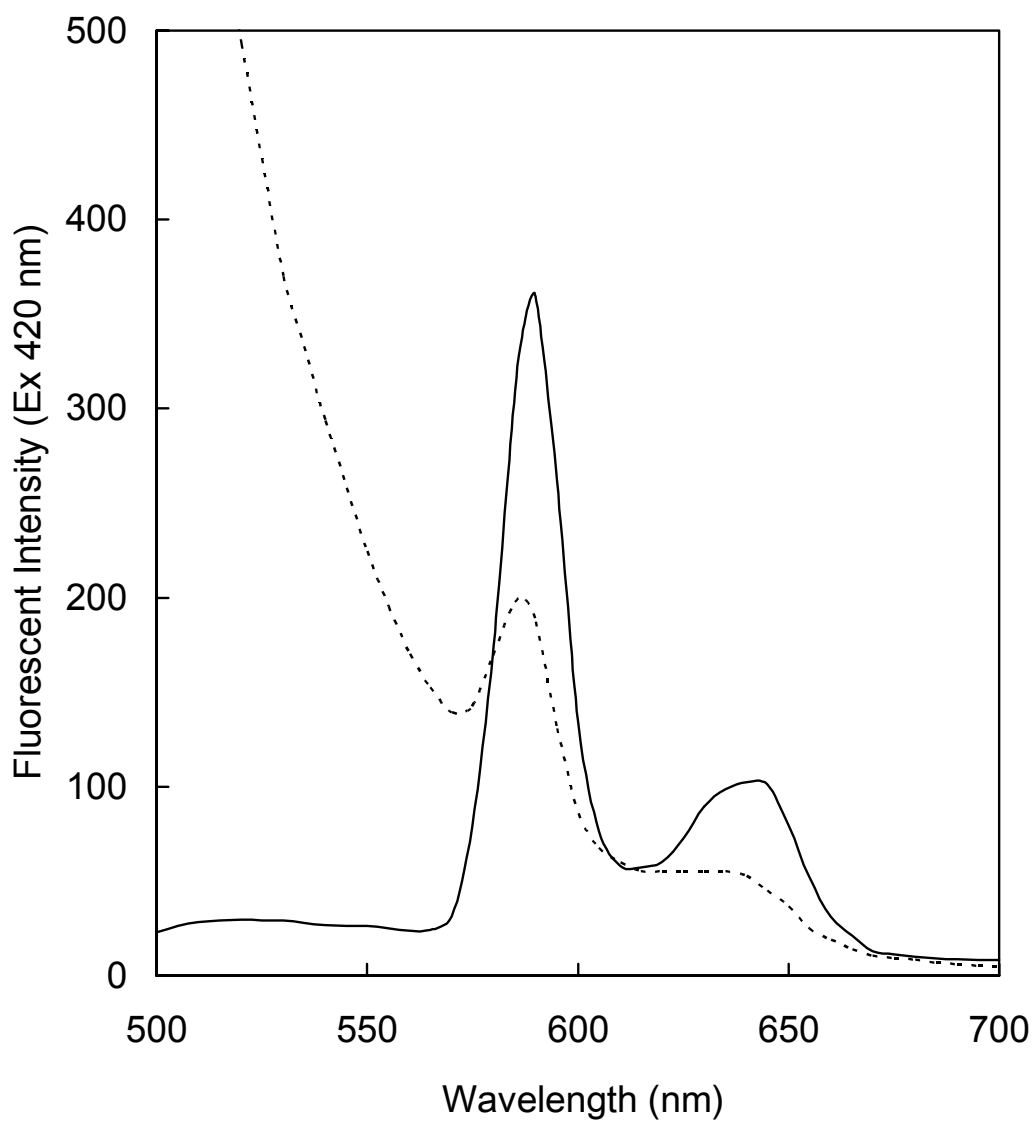
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13 Fig. 4. Fluorescence pattern of acetone extract of model solution after
14 anaerobic (—) and aerobic (----) incubation for 5 days at 25 °C with
15 antibiotics.

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17 Fig. 5. Effects of pre-heating of the meat homogenate on the formation of
18 ZPP before (0 days) and after anaerobic incubation (5 days) at 25 °C.

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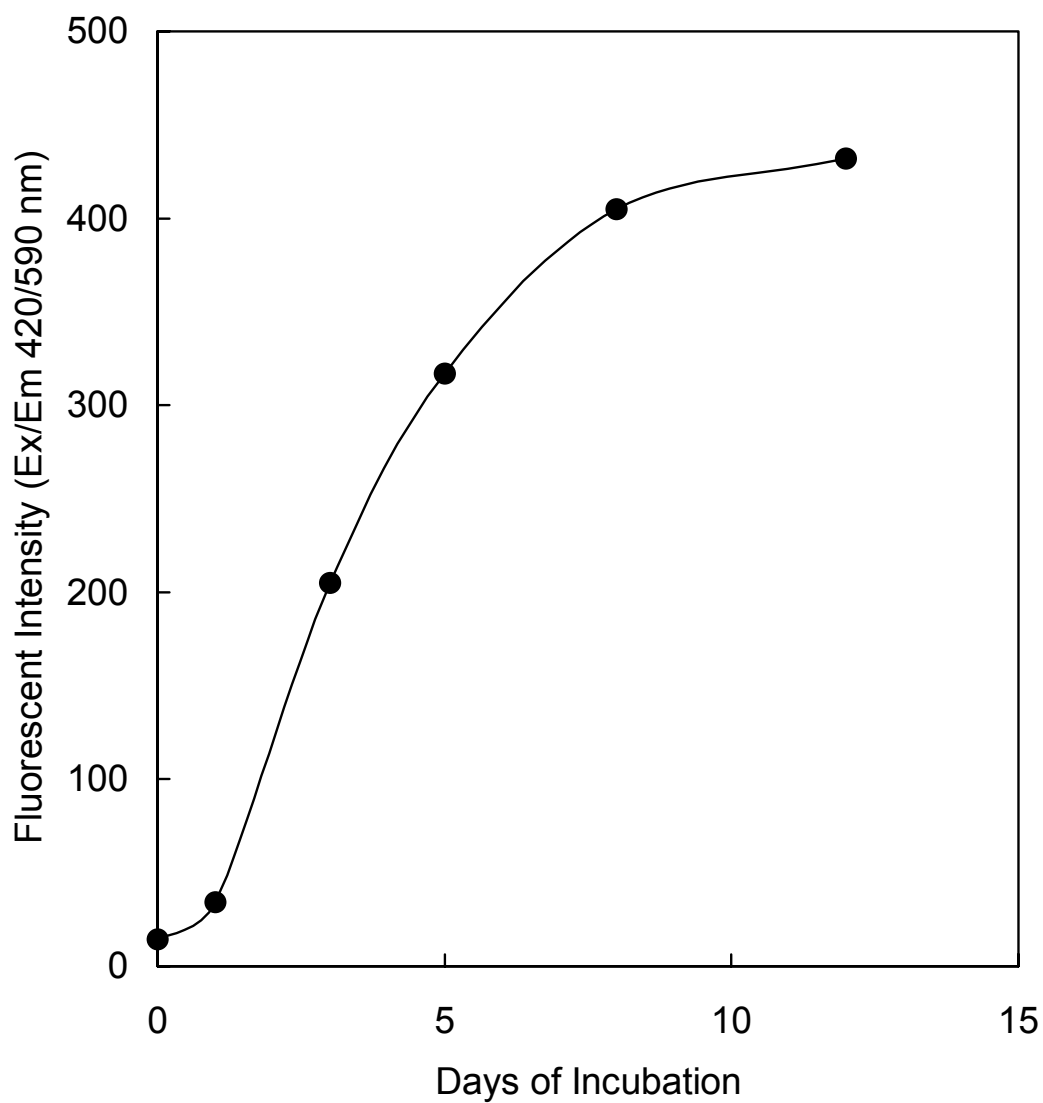
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Fig. 1.

7 Fluorescence pattern of acetone extract of model solution after anaerobic (—)

8 and aerobic (----) incubation for 5 days at 25 °C.

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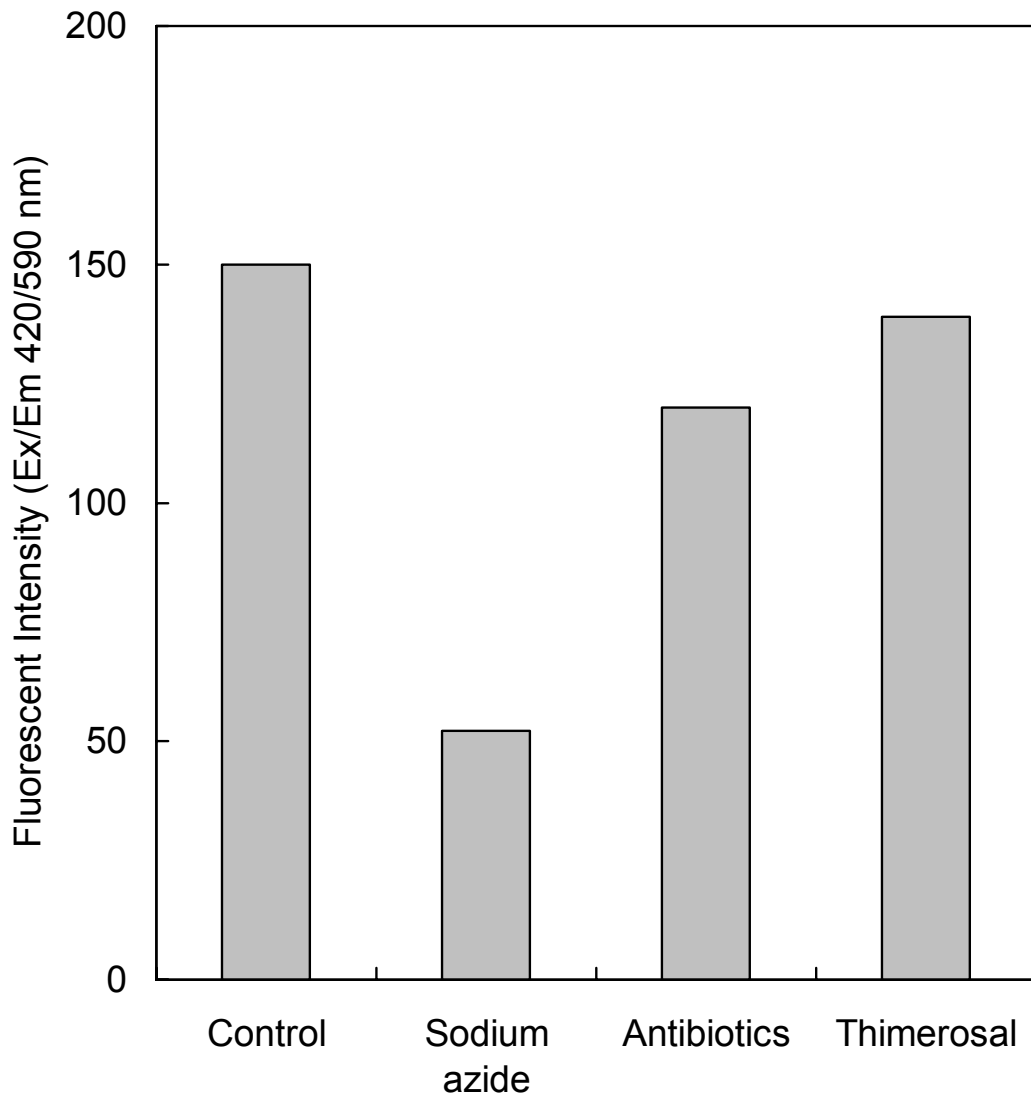
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5 Fig. 2.

6 Effects of anaerobic incubation time on the formation of ZPP.



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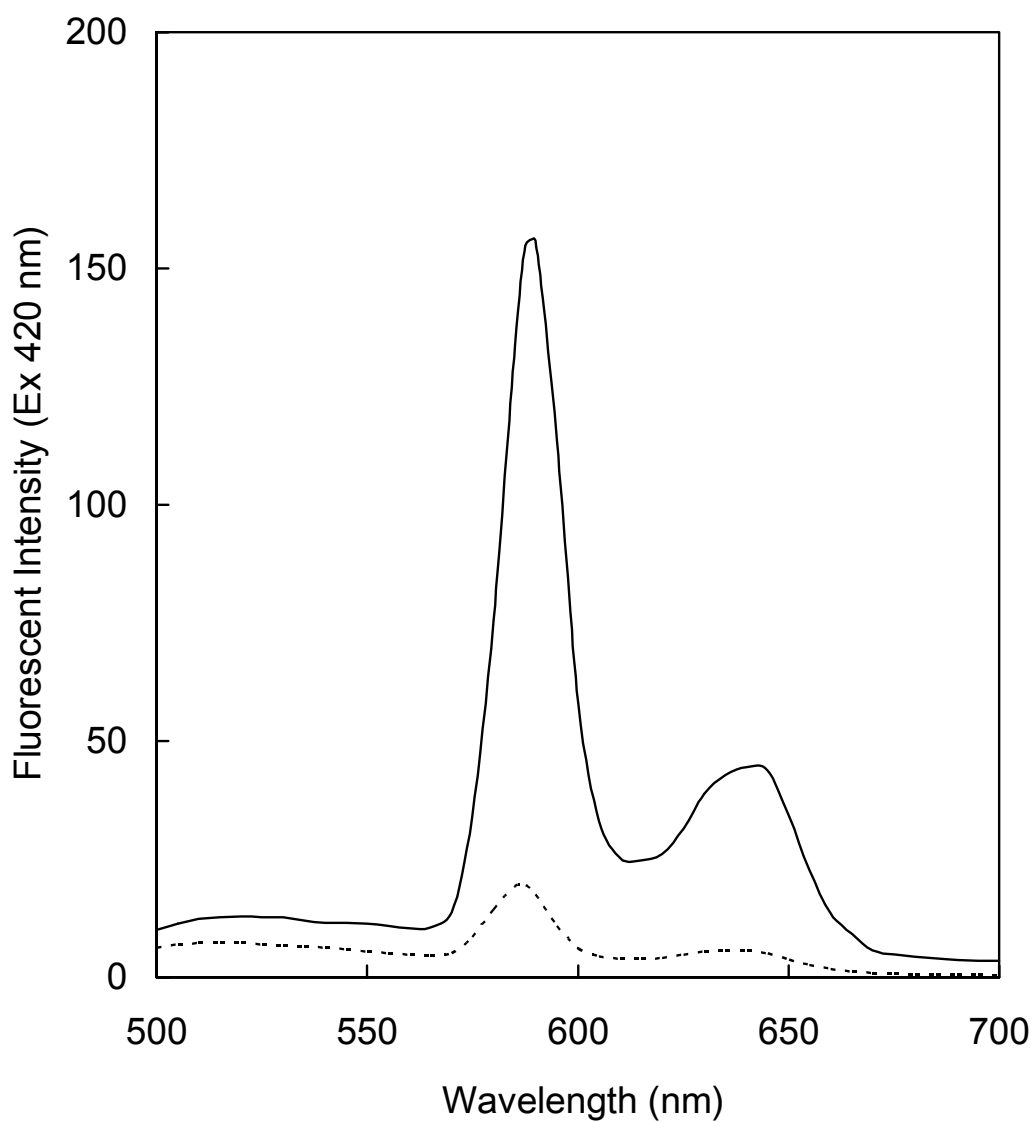
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6 Fig. 3.

7 Effects of various antiseptics (sodium azide, 0.01%; antibiotics [100 units/ml
8 penicillin G potassium, 0.1 mg/ml streptomycin sulfate and 0.05 mg/ml
9 gentamicin sulfate]; thimerosal, 0.01%) on the formation of ZPP after anaerobic
10 incubation for 5 days at 25 °C.

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Fig. 4.

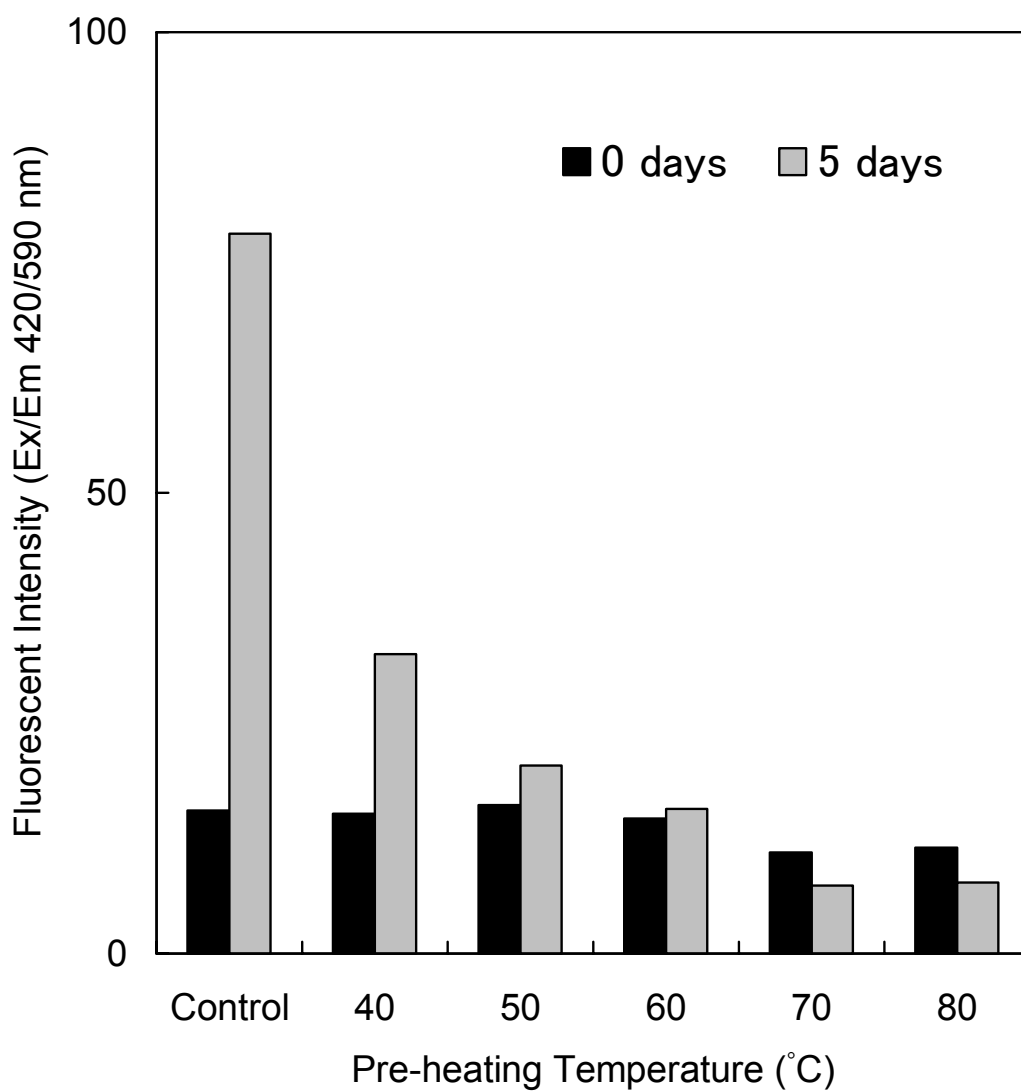
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Fluorescence pattern of acetone extract of model solution after anaerobic (—)

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and aerobic (----) incubation for 5 days at 25 °C with antibiotics.

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Fig. 5. Effects of pre-heating of the meat homogenate on the formation of ZPP before (0 days) and after anaerobic incubation (5 days) at 25 °C.